*VITAMIN D_3-STIMULATED TEMPLATE ACTIVITY OF CHROMATIN FROM RAT INTESTINE**

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Abstract.—Administration of vitamin D to rats deficient in this vitamin markedly increases the template activity for DNA-dependent RNA synthesis by rat intestinal mucosal chromatin. The maximum stimulation of template activity occurs three hours after a dose of 2000 international units of vitamin D_3 is given. These results support the concept that vitamin D functions by initiating the transcription of DNA into mRNA, which codes for functional protein(s) involved in calcium transport in the small intestine.

Introduction.—It is generally accepted that vitamin D stimulates the absorption of calcium in the small intestine and plays an important role in the mobilization of mineral from formed bone. These two physiologic actions are thought to work in concert to maintain the $[Ca^{++}]$ $[HPO_4^{=}]$ product in the blood at supersaturation levels with respect to bone mineral.[1]

Lund and DeLuca[2] first showed that a polar compound designated as peak IV which is a major biologically active metabolite of vitamin D appears in many tissues after ^{3}H-vitamin D administration. Stohs and DeLuca[3] found that four or eight hours after intravenous injection of 10 international units (IU) (0.25 μg) of ^{3}H-vitamin D_3, the major site of accumulation of radioactivity in rat and chick intestinal mucosa is the nuclear membrane, and that the predominant form of the vitamin in the target tissues (intestine and bone) is a polar metabolite. The peak IV metabolite of vitamin D_3 was recently identified by Blunt *et al.*[4] as 25-hydroxycholecalciferol (25-HCC); this is approximately 1.4 times as active as vitamin D_3 in curing rickets in rats and is also more active than vitamin D in chicks as determined by the bone ash assay (Blunt *et al.*[5]).

The need for conversion of vitamin D to the metabolically active form at least in part explains the lag in physiologic action after vitamin D administration. After a rat deficient in vitamin D is given 100 IU (2.5 μg) of vitamin D_3 intravenously, serum calcium does not begin to rise significantly for 8 to 12 hours. However, four to eight hours after 2.5 μg of 25-HCC is given, serum calcium is elevated (Blunt *et al.*[5]).

Recent experiments indicate that 25-hydroxycholecalciferol may be the sole active form of vitamin D_3 in the target tissues. Trummel *et al.*[6] have shown that 0.9 IU/ml of 25-HCC stimulates mobilization of calcium from isolated embryonic bone, whereas 380 IU/ml vitamin D_3 does not. In addition, Olson and DeLuca[7] have shown that there is a very rapid rise in calcium transport in a perfused rat intestine after treatment *in vitro* with 2.5 μg of 25-HCC, whereas 500 μg vitamin D_3 has no effect.

In 1964, Eisenstein and Passavoy[8] found that actinomycin D, an inhibitor of DNA-directed RNA synthesis, blocks the hypercalcemic response to large doses

of vitamin D. Zull *et al.*[9] showed that if actinomycin D is given one hour prior to vitamin D, there is a complete blockage of serum calcium elevation, of increased transport of calcium by everted gut sacs, and of increased *in vivo* calcium absorption. However, when this antibiotic is given four hours after vitamin D, there is no inhibition of the physiologic responses. Puromycin and 5-fluororotic acid also were found to block vitamin D action. Subsequently, Stohs *et al.*[10] found that an intraperitoneal dose of 2000 IU of vitamin D_3 gives two- to three-fold enhancement of the incorporation of ^{3}H-orotic acid into nuclear RNA of intestinal mucosa, but not into mitochondrial or ribosomal RNA. The enhancement is sensitive to actinomycin D. Maximal incorporation occurred three hours after administration of 2000 IU and five to eight hours after 10 IU of vitamin D_3.

These results suggested that vitamin D acts in the intestine by stimulating DNA transcription into mRNA, which in turn is translated into protein(s) functional in the calcium transport system. It therefore is important to establish that vitamin D exerts a direct effect on the genetic activity of intestinal mucosa. It is the purpose of this report to show that vitamin D markedly increases the template activity for DNA-directed RNA synthesis in isolated chromatin from rat intestine.

Methods.—Male weanling rats (Holtzman Co., Madison, Wisconsin, or Sprague-Dawley Co., Madison, Wisconsin) were maintained in individual, hanging wire cages and were given food and distilled water *ad libitum*. They were fed a diet adequate in calcium (0.47%) and phosphorus (0.3%), as previously described.[11] After 3 to 4 weeks the rats were deficient in vitamin D as evidenced by lowered serum calcium and reduced growth. They were used for experiments after 4 to 5 weeks.

Rats deficient in vitamin D were given intraperitoneally a single dose of 2000 IU vitamin D_3 in 0.5 ml of aqueous solution containing 0.9% NaCl and 0.1% Tween 40. The rats were killed 2, 3, or 4 hr after treatment. Control animals deficient in vitamin D were given an intraperitoneal injection of the aqueous vehicle and killed at the same times as the animals treated with vitamin D. The mucosa was isolated from the first 25 cm of the small intestine as previously described.[10] Chromatin was prepared from the pooled mucosa of two rats by the method of Marushige and Bonner.[12]

The isolated chromatin was used as a template for DNA-directed RNA synthesis in an *in vitro* RNA-synthesizing system with the use of highly purified *Escherichia coli* DNA-dependent RNA polymerase prepared by the method of Burgess;[13] chromatography on phosphocellulose was employed for final purification. The amount of RNA synthesized was measured by the incorporation of 8-[^{14}C]-ATP (Schwarz BioResearch) into acid-insoluble RNA. The RNA synthesized with a chromatin template, and *E. coli* RNA polymerase has the complementary base sequence to the DNA template and is generally similar to the RNA synthesized *in vivo*.[14]

Results.—The chromatin from animals treated with 2000 IU of vitamin D_3 three hours before they were killed has a markedly higher template activity than that from control-treated animals, when measured over a range of chromatin concentrations (Fig. 1).

In four experiments the template activity of the chromatin from rats receiving 2000 IU of vitamin D_3 three hours before they were killed was 1.3 to 2.8 times as high as that of the controls (Table 1). When the template activity is measured at various times after the administration of this dosage of vitamin D_3, the maximum response occurs at three hours (Table 1). When vitamin D is given two

TABLE 1. *Enhancement of template activity of chromatin from rat intestine at various times after administration of 2000 IU of vitamin D_3.*

Expt.	Time of exposure to vitamin D	AMP incorp. ($\mu\mu$M/ml with 10 μg DNA)	Ratio AMP incorp. in vitamin D-treated vs. control
1	Control	336	—
	3 Hr	456	1.4
2	Control	320	—
	2 Hr	360	1.1
	3 Hr	620	1.9
	4 Hr	480	1.5
3	Control	600	—
	3 Hr	800	1.3
	4 Hr	750	1.2
4	Control	142	—
	3 Hr	400	2.8
	4 Hr	142	1.0

The template assay is described in the legend to Fig. 1; 10–30 μg RNA polymerase was used. The time of exposure of the rat to 2000 IU of vitamin D_3 is given in the second column. Chromatin from each preparation was incubated at three to five concentrations in each experiment. From a plot of AMP incorporation vs. chromatin concentration, the template activity with a chromatin concentration of 10 μg DNA/ml is determined, and this value is given in the third column. The enhancement of template activity is expressed in the fourth column as the ratio of template activity of chromatin from the vitamin D-treated rats over the control rats.

hours before the rats are killed, the template activity is not yet enhanced and the response of template activity to a four-hour exposure to vitamin D is less than to a three-hour exposure.

When RNA polymerase or chromatin is omitted from the incubation mixture, no measurable RNA is produced (Fig. 1).

Discussion.—The present experiments provide direct evidence that vitamin D acts on the chromatin of intestinal mucosa to stimulate transcription of DNA into RNA. Its action is to "unmask" or increase the template activity of the chromatin for added RNA polymerase. Exactly how vitamin D increases the template activity is not known, although Stohs and DeLuca[3] demonstrated that the nuclear membrane of intestinal mucosa is a major site of ^3H accumulation from ^3H-vitamin D_3. Others[15] have reported that ^3H from ^3H-vitamin D_3 is

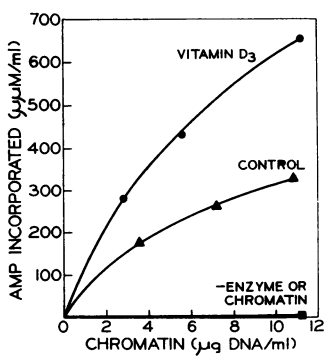

FIG. 1.—Enhancement by vitamin D (2000 IU) of template activity of chromatin from rat intestinal mucosa.

The reaction mixtures containing chromatin, 0.04 *M* Tris pH 7.9, 0.15 *M* KCl, 0.0046 *M* $MgCl_2$, 0.002 *M* $MnCl_2$, 0.07 mM EDTA, 5.8 mM β-mercaptoethanol, 0.5 mg/ml bovine serum albumin, 0.15 mM UTP, CTP, GTP, and 0.15 mM 8-[^{14}C]-ATP, and 20 μg *E. coli* RNA polymerase in 0.25 ml were incubated 10 min at 37°C. Reaction was stopped by adding 2 ml ice-cold 5% TCA. RNA was collected on Whatman GF/C filters which were washed 4 times with 5% TCA followed by 2 washings with ETOH. Filters were dried and counted under 10 ml toluene counting solution (0.01% dimethyl POPOP and 0.2% PPO in toluene) in Packard Tri-Carb liquid scintillation counter.

(—●—) Vitamin D_3 injected i.p. 3 hr before killing of rats; (—▲—) control vehicle injected 3 hr before killing of rats; (—■—) either chromatin preparation without RNA polymerase or RNA polymerase without chromatin.

located in isolated chromatin, but we have been unable to confirm this report. If vitamin D or its "active form" is located in the nuclear membrane, there may be several possible mechanisms to explain how vitamin D controls DNA transcription into RNA. Calcium ions might act as the specific inducer and/or repressor of the mRNA. Alternatively, the vitamin D-active compound might act directly or together with a receptor protein as an inducer of DNA transcription. The present system promises to provide new insight into this important problem.

The concept that vitamin D in some way induces transcription of a specific DNA in the intestine which codes for a calcium transport component(s) was based on the findings (1) that actinomycin D completely blocks the action of vitamin D, and (2) that vitamin D stimulates the pulse labeling of nuclear RNA by ^{3}H-orotic acid in the small intestine. The effect of vitamin D on the RNA labeling (three hours after the injection of 2000 IU of vitamin D_3) precedes the appearance of the calcium transport system by about three hours. The present experiments are in exact agreement, because increased template activity of isolated chromatin is maximal three hours after injection of 2000 IU of vitamin D_3. These data strongly suggest that the increased RNA labeling observed *in vivo* arises from increased template activity of the chromatin. Work is now in progress to assess the effect of the active metabolite of vitamin D, 25-hydroxycholecalciferol, on the template activity *in vivo* and *in vitro*. Clearly, this material acts more rapidly than vitamin D in stimulating intestinal calcium transport and is most likely the metabolically active form of the vitamin. Preliminary results suggest that both RNA pulse labeling *in vivo* and template activity are increased within minutes after 25-HCC administration. Thus, it seems probable that increased template activity of intestinal chromatin is the earliest biochemical event initiated by vitamin D.

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